Immunobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Short communication

Dual requirement for STAT signaling in dendritic cell immunobiology

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ARTICLE INFO

Keywords:
Dendritic cell
STAT
Small molecule inhibitor
Cytokine

ABSTRACT

Dendritic cells (DC) represent an attractive target for therapeutic manipulation of the immune system and enhancement of insufficient immune response in cancer. STAT family members play key roles in the differentiation and activation of DC, a feature that is currently being exploited in DC-based therapies. We previously reported that the small-molecule Stattic, originally developed as a STAT3-specific inhibitor, also inhibits STAT1 and STAT2 phosphorylation in DC exposed to cytokines or LPS. Aim of this study was to investigate the functional consequences of in vitro treatment with Stattic on DC immunobiology. Interestingly, we observed an opposite effect of Stattic on DC immunophenotype depending on the activation state. While the expression of costimulatory, coinhibitory, MHC class II and CD83 molecules was enhanced in immature DC exposed to Stattic, the LPS induced up-modulation of these molecules was strongly repressed. An effective blockade of LPS-induced secretion of proinflammatory cytokines and capacity to stimulate a Th1 polarization was also observed in the presence of Stattic. Our results indicate that the immunological consequences of STAT inhibition in DC vary depending on the cell activation state. This knowledge is of relevance for anticipating potential effects of STAT-targeted therapeutics, and pursuing selective DC manipulation in clinical applications.

1. Introduction

Dendritic cells (DC) shape the magnitude and duration of immune responses and play a pivotal role in controlling the balance between immune activation and immunological tolerance (Banchereau et al., 2003; Steinman, 2007). They are the most potent antigen presenting cells (APC) and orchestrate $CD4^+$ T helper (Th) and $CD8^+$ cytotoxic responses as well as T regulatory responses. In recent years, much work has been carried out to exploit the unique features of DC in clinical applications, especially in cancer immunotherapy (Bol et al., 2016).

STAT transcription factors mediate cytokine and growth factor signaling, and are critical for initiation, regulation and termination of immune responses. STATs have important roles in controlling both the development and the activation of DC. STAT3 in particular, is a critical regulator of DC physiology. It mediates the Flt3L-dependent development and the expansion of the DC compartment (Esashi et al., 2008; Laouar et al., 2003; Onai et al., 2006), and also acts as a negative regulator of the DC immunostimulatory potential. This latter function has been mainly investigated in the context of antitumor immunity, since STAT3 is constitutively activated in different tumors as well as in tumor-infiltrating immune cells. In mouse models, STAT3 hyperactivation in tumor-infiltrating DC impairs their differentiation and functional maturation (Kortylewski et al., 2005; Nefedova et al., 2004; Wang et al., 2004). Accordingly, the inhibition of Jak2/STAT3 signaling dramatically improves differentiation and activation of murine DC (Nefedova et al., 2005). STAT3 ablation in hematopoietic cells from adult mice enhances DC maturation in tumor-bearing mice (Kortylewski et al., 2005) and conditional KO mice with STAT3 deletion in $CD11c^+$ DC (Melillo et al., 2010) exhibit an altered immune homeostasis and chronic inflammation. In humans, we and others have shown that silencing STAT3 in monocyte-derived DC enhanced their capacity to prime Th1 responses (Iwata-Kajihara et al., 2011;

Abbreviations: APC, antigen presenting cells; CSFE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cells; FBS, fetal bovin serum; iDC, immature DC; LPS, lipopolysaccharide; mDC, mature DC; MLR, mixed leucocyte reaction; mAb, monoclonal antibodies; PBMC, peripheral blood mononuclear cells; RNAi, RNA interference; Th, T helper; TLR, toll-like receptor

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http://dx.doi.org/10.1016/j.imbio.2017.10.049
Received 21 June 2017; Received in revised form 26 October 2017; Accepted 26 October 2017

Sanseverino et al., 2014). Conversely, STAT1 activation was shown to be important for full DC maturation and Th1 cell priming, both in the context of the response to microorganisms and in the tumour microenvironment. STAT1 activation in DC is required for induction of MHC and co-stimulatory molecules as well as antigen presentation, including cross-presentation to activate $CD8^+$ T cells (Diamond et al., 2011; Johnson and Scott, 2007; Pilz et al., 2009). STAT1-depleted DC elicit deficient immune responses to *Listeria monocytogenes* and *Leishmania major*, due to impaired Th1 cell priming or an increase in antigen-specific T regulatory cells (Johnson and Scott, 2007; Kernbauer et al., 2012). Very recently, an IL-33-STAT1 axis has been described that restores myeloid DC activation and maturation and the magnitude of antitumor immune responses in established cancer models (Dominguez et al., 2017). Finally, together with STAT2, STAT1 is the main STAT involved in transducing type I IFN signaling, which plays pivotal roles in modulating DC activation and functions and their ability to orchestrate immune responses to malignant cells in the tumor microenvironment (Cheon et al., 2014).

Stattic is a small-molecule STAT inhibitor, identified in a screening of chemical libraries for its ability to compete with a high-affinity phosphopeptide targeted to the SH2 domain of STAT3 and selected for its specificity to inhibit IL-6 driven STAT3 activation (Schust et al., 2006). We reported that – at least in DC – Stattic has the potential to also inhibit STAT1 and STAT2 phosphorylation induced by cytokines (i.e. type I IFN) or by lipopolysaccharide (LPS) stimulation (Sanseverino et al., 2012). Likewise, inhibition of STAT1 phosphorylation by Stattic was described in different cell models such as human ovarian cancer and melanoma cells (Bill et al., 2010; Debnath et al., 2012). Due to the well described role of STAT3 in cancer development and immune escape, Stattic has received particular attention as a candidate drug for the treatment of human tumors (Han et al., 2014; Ji et al., 2013; Lin et al., 2016; Pan et al., 2013; Zhang et al., 2015). However, the effects of Stattic on DC biology have been little explored in spite of the relevance of DC in the antitumor response and therapy. Here, we investigated the functional consequences of in vitro exposure of DC to Stattic by analyzing the immunophenotype, the secretory profile and the T cell polarization activity of both immature DC (iDC) andLPS-matured DC (mDC).

2. Material and methods

2.1. Cell isolation and culture

Monocytes were isolated from peripheral blood of healthy donors by Ficoll-Paque density centrifugation followed by immunomagnetic selection with $CD14^+$ microbeads (MACS monocyte isolation kit from Miltenyi Biotec). To obtain iDC, monocytes were seeded at 1×10^6 cells/ml in RPMI medium (BioWhittaker) supplemented with 2 mM L-glutamine, 2 mM penicillin/streptomycin and 10% fetal bovin serum (FBS, Hyclone) in the presence of GM-CSF (50 ng/ml; Shering Plough) and IL-4 (500 IU/ml; Shering Plough). Cytokines were added to the culture every 3–4 days. To obtain mDC, cells were stimulated at day 6 with LPS (serotype EH100, Ra TLR grade, Alexis Biochemicals, 10 ng/ml) for 24 h (hr). Stattic (Sigma-Aldrich) was used at the indicated concentrations (2 and 10 μM) for 25 h, and added to the cells 1 h before LPS.

2.2. Flow cytometric analysis

For the analysis of the immunophenotype DC were preincubated for 30 min on ice with PBS containing 10% human AB serum to block nonspecific Ig binding and then incubated with the specific monoclonal antibodies (mAbs) or the control isotypes for 30 min on ice, washed and analyzed with a FACSCalibur flow cytometer (BD Biosciences) using the CellQuest program. Cell staining was performed by using the following fluorocrome-conjugated mAbs: CD1a (-FITC, BD Pharmigen), CD14, CD80, CD83, CD86, HLA-DR and B7H-1 (-PE, BD Pharmigen), ILT3 and ILT4 (-FITC, R & D System).

2.3. Cytokine and chemokine determination in culture supernatant

Culture supernatants were collected and frozen at −20 °C until analyzed by ELISA for the content of the following cytokines and chemokines: TNF-α, IL-10, IL-6 (BioLegend), IL-12 (R & D Systems) and IFN-γ (eBioscience) according to manufacturer's instructions.

2.4. Type I IFN biological titration

Type I IFNs secreted in culture supernatants were measured using a cytopathic effect reduction assay with HeLa cells (1×10^4 cells/well in 96-well microplates) and vesicular stomatitis virus at a multiplicity of infection of 0.1 PFU/cell as challenge virus. Human IFN-α reference standard (Ga23- 902-530; National Institutes of Health, Bethesda, MD) was used at a dilution of 500 IU/ml. The sensitivity of the assay ranged from 5 to 15 IU/ml.

2.5. Mixed leukocyte reaction assay (MLR)

Naïve $CD4^+$ T cells were obtained from Ficoll-isolated peripheral blood mononuclear cells (PBMC) through naïve $CD4^+$ T cell isolation Kit II (Miltenyi Biotech). DC were stimulated at day 6 of culture with LPS or left untreated, and 24 h later co-cultured with allogenic naïve $CD4^+$ T cells, at a 1:10 ratio in RPMI 5% human AB pool serum (Lonza). To evaluate T cell expansion, $CD4^+$T cells to be used in the MLR assay were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as described (Quah et al., 2007). Briefly, freshly isolated cells were resuspended in PBS at a concentration of 1.5×10^6/ml in the presence of 5 μM CFSE (AlexisBiochemicals). After 4 min at room temperature, the labeling reaction was stopped by diluting with 20 vols of RPMI containing 10% FBS. Upon two washes in RPMI medium without serum, labeled cells were resuspended in RPMI containing 5% human AB pool serum and co-cultured with DC resuspended in the same medium. Cells were harvested after 5–7 days of co-culture and analyzed by flow cytometry. Supernatant were collected and frozen at −20 °C until analyzed by ELISA.

2.6. Statistics

Statistical analysis was performed by the Student's *t*-test. Probability p values ≤0.05 were considered to reflect statistical significance. Analyses were conducted using Graphpad (Version 6.05).

3. Results

3.1. Opposite effect of Stattic on the immunophenotype of immature and mature DC

We evaluated the effect of Stattic on the basal and LPS-induced expression of MHC (HLA-DR) and co-stimulatory molecules (Fig. 1A–C), inhibitory molecules and receptors (Fig. 1D–F), and lineage and maturation markers (Fig. 1G–I). Upon treatment with Stattic for 24 h, a dose-dependent increase in the percentage (black columns) and fluorescence intensity (grey columns) of iDC positive for the expression of HLA-DR and CD86 was observed. A trend toward enhanced expression of CD80 was also present. On the contrary, upon exposure to LPS, Stattic clearly inhibited the maturation-induced up-modulation of both HLA-DR and CD86 (Fig. 1A and B). A similar bimodal effect was observed for the expression of B7H1 (PDL1). Stattic induced an increase in both the percentage and fluorescence intensity of B7H1 positive iDC and a marked decrease of the LPS-induced B7H1 up-modulation (Fig. 1D).

We next analyzed the expression of ILT3 and ILT4, belonging to a